FEDEROFF DECLARATION

Exhibit E

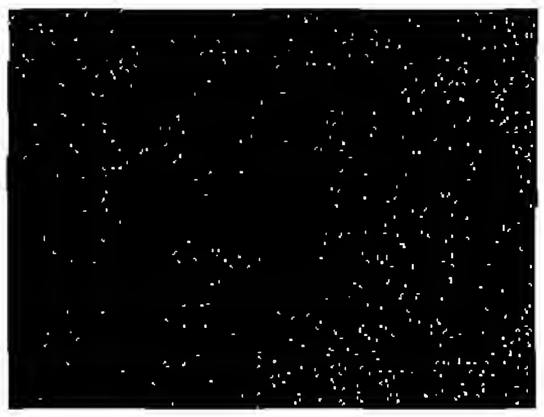

FIG. 4. Immunoblot of free and lipid-associated apo(*a*) from the plasma of transgenic mice on the atherogenic diet. Lane P: Total plasma (10 μl); lanes 1–5, lipid-free (density > 1.215 g ml^{-1}) fraction, adjusted to final volume of 500 μl: lane 1, 10 μl: lane 2, 7.5 μl; lane 3, 5 μl; lane 4, 2.5 μl, lane 5, 1.25 μl. Lane 6 contains 75 μl out of 500 μl of the lipoprotein fraction of density <1.215 g ml^{-1}.

METHODS. Pooled plasma (500 μl) from three apo(*a*) transgenic mice on the atherogenic diet was adjusted to a density of 1.215 g ml^{-1} with KBr and centrifuged. After adjusting to equal volumes, aliquots of the top and bottom fractions were electrophoresed on a 6% SDS–polyacrylamide gel under reducing conditions, transferred to nitrocellulose and probed with sheep anti-human apo(*a*) (Immuno, Vienna) at 1:400, followed with peroxidase-conjugated anti-goat IgG at 1:400, and colour development with 4-chloro-1-naphthol. The ratio of apo(*a*) in dilutions of the bottom (lipid-free) and top fractions was used to estimate that ~5% of the plasma apo(*a*) is lipid-associated.

susceptibility in mice[6]. This would not account for the differences seen here, in which control and transgenic groups have similar HDL levels. The highly significant differences in lesion areas between the groups, and the co-localization of apo(*a*) to the lesions in the transgenic animals, strongly indicate that the difference in atherogenesis between these two groups is due to apo(*a*). We cannot be certain how the expression of the apo(*a*) transgene triggers fatty streak formation. The significant 'atherogenesis' in the transgenic mice, despite the fact that only 5% of the plasma apo(*a*) is found in the lipid float, suggests that apo(*a*) could effect vessel pathophysiology independently of the apo B-100 and lipid portions of the Lp(*a*) lipoprotein. It is possible that the fraction of the transgenic apo(*a*) that is lipid-associated binds to sites in the vessel in the same way as it would in humans. Alternatively, free apo(*a*) might bind to components in the vessel wall for which it has affinity, such as fibrin, fibronectin, collagen, elastin, glycosaminoglycans, endothelial cells or macrophages[18–24], and this immobilized apo(*a*) might then enhance the deposition of lipoproteins by virtue of the high affinity of apo(*a*) for apoB-100-containing lipoproteins[25]. These results also indicate that the relationship of Lp(*a*) to atherosclerosis may result in part from binding of free apo(*a*) to sites in the vessel wall followed by antifibrinolytic[19,20,26–28], pro-cell migratory[29] or inflammatory activities, which could ultimately lead to hyperplasia and macrophage recruitment and the appearance of lipid-containing lesions. Further studies with transgenic animals expressing human apo(*a*) as well as other apolipoprotein genes are necessary to sort out these possibilities. □

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Mutation of the β-amyloid precursor protein in familial Alzheimer's disease increases β-protein production

Martin Citron†, Tilman Oltersdorf*†, Christian Haass, Lisa McConlogue*, Albert Y. Hung, Peter Seubert*, Carmen Vigo-Pelfrey*, Ivan Lieberburg* & Dennis J. Selkoe

Department of Neurology and Program in Neuroscience, Harvard Medical School, and Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, Massachusetts 02115, USA
* Athena Neurosciences Inc., South San Francisco, California 94080, USA

PROGRESSIVE cerebral deposition of the 39–43-amino-acid amyloid β-protein (Aβ) is an invariant feature of Alzheimer's disease which precedes symptoms of dementia by years or decades. The only specific molecular defects that cause Alzheimer's disease which have been identified so far are missense mutations in the gene encoding the β-amyloid precursor protein (β-APP) in certain families with an autosomal dominant form of the disease (familial Alzheimer's disease, or FAD)[1–5]. These mutations are located within or immediately flanking the Aβ region of β-APP, but the mechanism by which they cause the pathological phenotype of early and accelerated Aβ deposition is unknown. Here we report that cultured cells which express a β-APP complementary DNA bearing a double mutation (Lys to Asn at residue 595 plus Met to Leu at position 596) found in a Swedish FAD family[5] produce ~6–8-fold more Aβ than cells expressing normal β-APP. The Met 596 to Leu mutation is principally responsible for the increase. These data establish a direct link between a FAD genotype and the clinicopathological phenotype. Further, they confirm the relevance of the continuous Aβ production by cultured cells[6–8] for elucidating the fundamental mechanism of Alzheimer's disease.

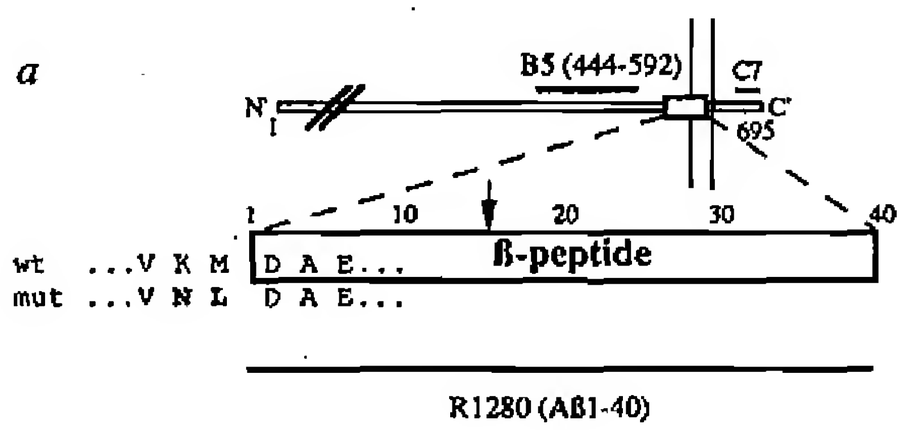
Human kidney 293 cells were transiently transfected with DNA constructs encoding either wild-type β-APP_{695} (ref. 9) or β-APP_{695} containing the Swedish FAD double mutation[5], in which the two amino acids immediately amino-terminal to the Aβ region (595 and 596 in β-APP_{695}, or 670 and 671 in β-APP_{770}) are changed (Fig. 1*a*). Subconfluent cultures of each transfected cell type were metabolically labelled with [^{35}S]methionine for 16 hours and the conditioned medium immunoprecipitated with a high-titre antibody (R1280) against synthetic $Aβ_{1-40}$ (ref. 6). Fluorography of the precipitates consistently showed substantially more Aβ in the medium from

† M.C. and T.O. made equal contributions to this work.

cells expressing the mutation (Fig. 1*b*). The Aβ peptide produced by both cell types had all of the characteristics recently described for *in vitro*-generated Aβ (ref. 6), including co-migration with synthetic $Aβ_{1-40}$ (Fig. 1*b*), greater production by β-APP-transfected than by mock-transfected cells (Fig. 2), and failure to immunoprecipitate with the amino-terminal β-APP antibody, B5, and the carboxy-terminal antibody, C7 (data not shown). The increase in Aβ production in the Swedish transfectants was accompanied by a decrease in the peptide of M_r 3,000 (3K) (known as p3), which starts at residue 17 or 18 of Aβ (ref. 6) and represents a fragment of the 10K carboxy-terminal peptide that arises from constitutive secretory cleavage of β-APP[10-14].

The increase in Aβ in the medium from the Swedish transfectants was quantitated using an Aβ-specific sandwich enzyme-linked immunosorbent assay (ELISA)[7]. The levels of Aβ detected by this assay were normalized to those of secreted soluble β-APP (APP_s[11-14]) detected in the same medium by a sandwich ELISA specific for APP_s (Fig. 2). Cells expressing the mutant $β\text{-}APP_{695}$ construct produced 6–7-fold more Aβ than identically transfected cells expressing wild-type $β\text{-}APP_{695}$ (Fig. 2). This marked increase was consistently obtained in all transfections with the mutant construct. Moreover, a 7–8-fold increase of Aβ was observed in cultures expressing the Swedish mutation in the $β\text{-}APP_{751}$ form of the precursor (Fig. 2). Similar increases in Aβ were found by phosphor-imager analysis of the 4K Aβ band in gels of R1280 immunoprecipates. This method further demonstrated that p3 was decreased several fold in the medium of the Swedish transfectants. We found that 293 and CHO cells stably transfected with the Swedish mutation in $β\text{-}APP_{751}$ also showed marked increases in Aβ in conditioned medium (data not shown).

To examine the mechanism for this increased Aβ production, we investigated the effects of each of the two mutations separately. Cells expressing $β\text{-}APP_{695}$ containing the 596 Met→Leu substitution had increased levels of Aβ in their medium, whereas cells expressing the 595 Lys→Asn substitution had levels similar to wild-type transfectants (Fig. 3, lanes 2–5). This finding suggests that the mutation at 596 is responsible for producing more proteolytic cleavage at the Leu–Asp peptide bond than at the normal Met–Asp bond. It is possible that the Lys→Asn switch at position 595 may further enhance the cleavage when coupled with the Met→Leu substitution. Next we created a DNA construct combining the Swedish double mutation with a deletion of almost all of the cytoplasmic domain of β-APP, thus removing the Asn–Pro–Xxx–Tyr consensus sequence[15] for coated pit-mediated internalization of cell-surface proteins which may direct the reinternalization of β-APP from the cell surface and its targeting to late endosomes/lysosomes[16]. Cells transfected with this construct still produce substantially more Aβ in their media



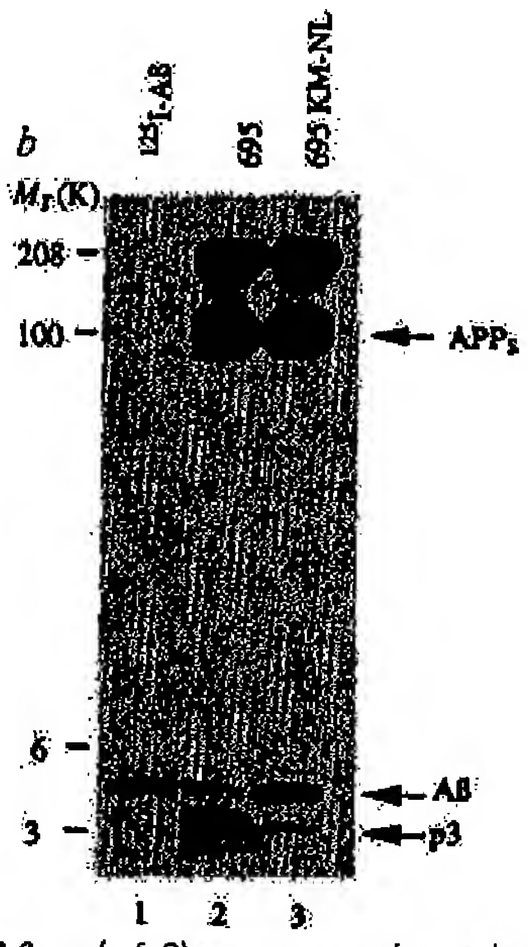


FIG. 1 Immunoprecipitation of Aβ from medium of cells transfected with wild-type or Swedish mutant $βAPP_{695}$ constructs. *a*, Schematic of β-APP and the Aβ region within it. Solid horizontal lines represent the regions against which antibodies B5, C7 and R1280 were raised; vertical lines designate the single membrane-spanning region of β-APP. Arrow indicates the constitutive secretory cleavage site of β-APP[13]. The amino-acid sequences of wild-type and mutant β-APP around the N terminus of Aβ are indicated. *b*, Conditioned medium of radiolabelled kidney 293 cells transiently transfected with either $β\text{-}APP_{695}$ (ref. 10) (lane 2) or $β\text{-}APP_{695}$ KM–NL (595 Lys→Asn +596 Met→Leu) (lane 3) were immunoprecipitated with R1280, ^{125}I-labelled synthetic $Aβ_{1-40}$ (ref. 6) was run as a size marker on the same gel (lane 1). The Aβ and p3 bands[6] are indicated by arrows. METHODS. A cDNA construct encoding $β\text{-}APP_{695}$ KM–NL as described[5] was designed by replacing the 26-base-pair *Bgl*II–*Eco*RI fragment of $βAPP_{695}$ with the annealed oligonucleotides GATCTCTGAAGTGAATCTGGATGCAG and AATTCTGCATCCAGATTCACTTCAGA. Transient transfections into 293 cells were performed using lipofectin (Gibco, BRL) as described by the manufacturer. Metabolic labelling with ^{35}S-methionine and immunoprecipitations with R1280 were done as described[6]. The immunoprecipitates were separated on a 10–20% Tris–Tricine gel, and autofluorographed for 9 days. The high-molecular-weight band (~218K) in lanes 2 and 3 is nonspecific and is variably precipitated with preimmune serum[8].

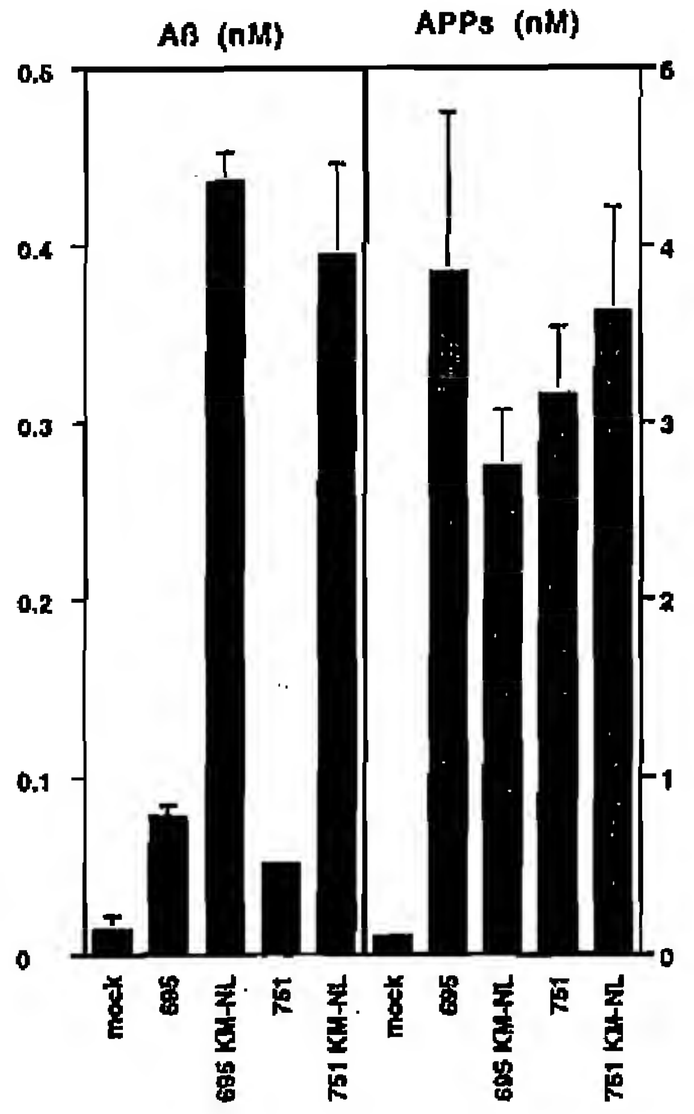


FIG. 2 Quantitation of Aβ (left panel) and APP_s (right panel) in conditioned medium of transiently transfected 293 cells using two distinct sandwich ELISAs. Each column represents the mean of four transfections, with the exception of the mock column which is based on three cultures. Error bars indicate the standard deviation. For columns without error bars, the standard deviation was less than 0.01.
METHODS. The sandwich ELISA for Aβ was done as before[7] with monoclonal antibodies 266 (against $Aβ_{13-28}$) and 6C6 (against $Aβ_{1-16}$). The sandwich ELISA for APP_s was similarly constructed with affinity-purified polyclonal antibodies, using capture antibody B3 against a bacterial fusion protein of $β\text{-}APP_{20-304}$ and reporter antibody B5 (biotinylated) against a bacterial fusion protein of $β\text{-}APP_{444-592}$ (ref. 12) ($βAPP_{695}$ numbering). For each ELISA, increasing amounts of purified synthetic $Aβ_{1-40}$ or purified APP_s from the conditioned medium of 293 cells transfected with $β\text{-}APP_{695}$ were used to construct a standard curve.

than wild-type transfectants, but levels of p3 are increased (Fig. 3, lanes 6 and 7). This result indicates that the effect of the Swedish mutations does not require an intact cytoplasmic domain and that generation of Aβ is unlikely to require processing of β-APP in late endosomes/lysosomes, a conclusion consistent with other recent results[17].

The findings reported here provide, to our knowledge, the first experimental evidence that point mutations in the β-APP gene found in FAD kindreds can result directly in increased generation of the Aβ peptide. To date, six β-APP missense mutations have been identified in families with autosomal dominant Alzheimer's disease[1-5]. In those cases in which the neuropathological phenotype has been documented, there is an early onset of progressive and severe β-amyloid deposition, particularly in the cerebral cortex. In the case of the Swedish family with the double mutations, the onset of clinical symptoms occurs at around 55 years of age[5]. Two different assays used here consistently demonstrate a marked increase in Aβ production by cells expressing the mutant precursor. A comparable decrease occurs in the production of the p3 peptide that begins at $Aβ_{17-18}$, the site of constitutive secretory cleavage of β-APP[13]. Several lines of evidence, including pulse-chase experiments, isolation of lysosomes, the use of drugs affecting protein processing, and the deletion of the putative internalization signal in the cytoplasmic domain, all suggest that p3 derives from the 10K C-terminal stub of β-APP after secretion of APP_s[17]. The increase in p3 seen in the ΔC constructs (Fig. 3, lanes 6 and 7) presumably derives from a decrease in reinternalization of these truncated β-APP molecules to lysosomes, with consequent increased secretory cleavage occurring at or near the cell surface.

The increase in Aβ and the corresponding decrease in p3 in the Swedish transfectants suggests that Aβ may arise after an alternative secretory cleavage that generates the AβN terminus. A shorter form of APP_s, which this alternative cleavage would produce, has recently been described[18]. The lack of attenuation of Aβ production by cells expressing the Swedish β-APP construct with a cytoplasmic deletion supports such an alternative secretory mechanism, rather than the generation of Aβ as a result of internalization of β-APP to an endosomal/lysosomal pathway, although we cannot yet rule out Aβ generation in early endosomes. This issue is now being addressed by developing antibodies that specifically recognize the last few residues of the shorter APP_s form including the substituted amino acids at 595 and 596 (ref. 18), and establishing whether such a secreted form increases in parallel with the rise of Aβ in the medium of the Swedish transfectants.

The double mutation of $β\text{-}APP_{595}$ and $β\text{-}APP_{596}$ consistently raises the level of Aβ *in vitro*, and the substitution of Leu for Met at 596 appears to be mainly responsible for this effect. It is important to determine whether the mutations at residue 717 of $β\text{-}APP_{770}$ immediately following the Aβ region[1-3] and those within the Aβ region[4,19] also raise Aβ production. It is likely that these and other mutations will be found to affect Aβ production to different degrees and by different mechanisms. Additional artificial mutations can also be examined *in vitro* to define the amino-acid specificities of the proteases that cleave at the N and C termini of Aβ. Analysis of the Swedish mutations demonstrates the advantage of measuring Aβ production *in vitro*[6-8] in transfected or primary cells bearing a particular mutation as a route to elucidating the specific mechanisms of accelerated β-amyloidosis in some familial forms of Alzheimer's disease. It will be interesting to see whether this marked rise in Aβ production *in vitro* is reflected in a change in the levels of the peptide in the body fluids of the Swedish patients. Moreover, β-APP-transfected cells or endogenous cells (such as fibroblasts) taken from a patient with the mutation could prove to be useful as a screen to identify compounds that lower cellular Aβ production. □

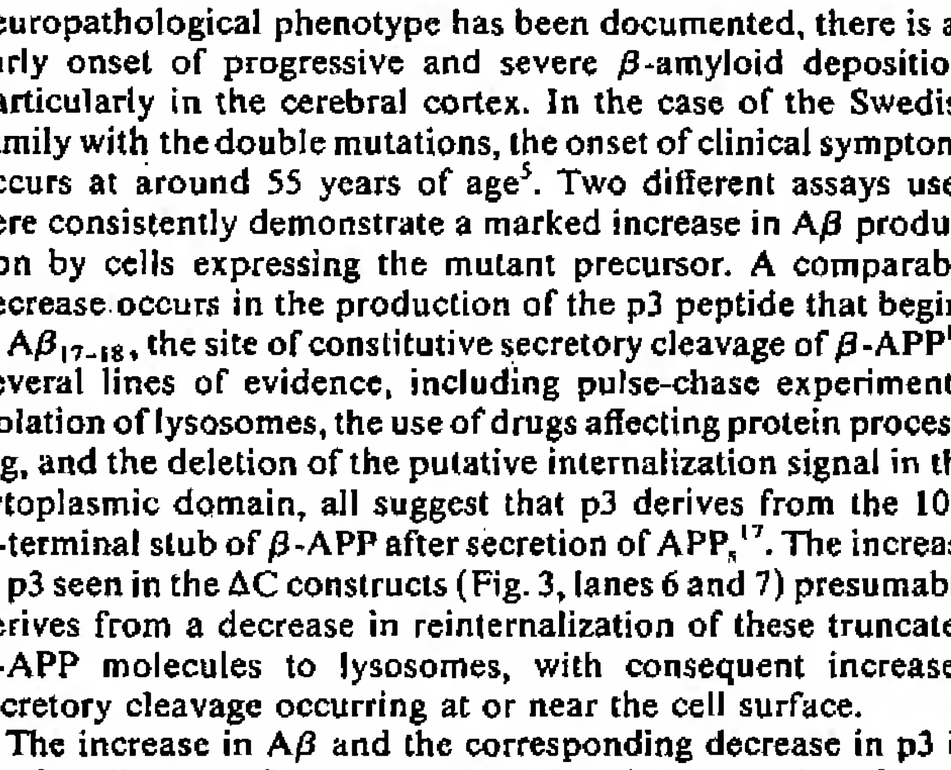


FIG. 3 Immunoprecipitation of Aβ from medium of cells transfected with various normal or mutant $β\text{-}APP_{695}$ constructs. Conditioned medium of radiolabelled kidney 293 cells transiently transfected with no DNA (lane 1), $β\text{-}APP_{695}$ (lane 2), $β\text{-}APP_{695}$ KM–NL (lane 3), $β\text{-}APP_{695}$ K–N (595 Lys → Asn; lane 4), $β\text{-}APP_{695}$ M–L (596 Met → Leu; lane 5), $β\text{-}APP_{695}ΔC$ (cytoplasmic deletion; lane 6) and $β\text{-}APP_{695}ΔC$ KM–NL (lane 7) were immunoprecipitated with R1280. ^{125}I-labelled synthetic Aβ (1–40) was run as a size marker on the same gel (lane 8). The Aβ and p3 bands[6] are indicated by arrows.
METHODS. cDNA constructs encoding $β\text{-}APP_{695}$ K–N and $β\text{-}APP_{695}$ M–L were constructed as described for $β\text{-}APP_{695}$ KM–NL (see Fig. 1) using the annealed oligonucleotides GATCTCTGAAGTGAATATGGATGCAG, AATTCTGCATCCATATTCACTTCAGA and GATCTCTGAAGTGAAGCTGGATGCAG, AATTCTGCATCCAGCTTCACTTCAGA, respectively. A construct encoding $β\text{-}APP_{695}ΔC$ KM–NL was engineered from $β\text{-}APP_{695}ΔC$ (ref. 17) using the strategy described in Fig. 1. Transfections and R1280 immunoprecipitations were as for Fig. 1.

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Projection domains of MAP2 and tau determine spacings between microtubules in dendrites and axons

J. Chen*, Y. Kanai*, N. J. Cowan† & N. Hirokawa*‡

* Department of Anatomy and Cell Biology, School of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan
† Department of Biochemistry, New York University, Medical Center, New York, New York 10016, USA

NEURONS develop a highly polarized morphology consisting of dendrites and a long axon. Both axons and dendrites contain microtubules and microtubule-associated proteins (MAPs) with characteristic structures[1]. Among MAPs, MAP2 is specifically

‡ To whom correspondence should be addressed.